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An Olfactory Based Behavioral Analysis of Bombus terrestris in Relation to Three Strains of Solanum lycopersicum

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AN OLFACTORY BASED BEHAVIORAL ANALYSIS OF *BOMBUS TERRESTRIS* IN RELATION TO THREE STRAINS OF *SOLANUM LYCOPERSICUM*

By

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A Thesis

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Abstract

Every year agricultural companies produce new strains of *Solanum*, the genus that includes species such as tomatoes, potatoes and eggplants. Using artificial selection, the strains are created for disease resistance and hardiness. However, it is unknown if pollinator-attractive traits are inadvertently lost by this process. It has been documented in strawberries that different strains of the same species produce different amounts of volatile organic compounds. The strain that produced the highest quantity of volatile organic compounds attracted the most pollinators under field conditions, and elicited the greatest antennographic response in Red Mason bees (Klatt et al., 2013). Therefore, I asked if bumblebees, one of the main pollinators of *Solanum*, are differentially attracted to multiple strains of tomatoes, an important cash crop. Of the three strains used in this study, I predicted that bumblebees would be most attracted to the cultivar with the least disease resistance because it was less likely that any attractive traits had been bred out. I tested this hypothesis using an olfactory based Y-maze behavioral apparatus and determined that in a laboratory setting bumblebees were significantly more attracted to Brandywine, the least disease resistant strain, than Mountain Magic, the cultivar with the greatest disease resistance profile. The results were confirmed using both cuttings of the plants and whole plants. From these data it is possible to say that the volatile organic compound profile of Mountain Magic may have been inadvertently modulated by artificial selection towards disease resistance. Therefore, it is necessary to create a larger scale study to determine if this trend is endemic to all artificially selected tomatoes, or localized to these two strains.

Background Article Review

General Information

Bumblebees are social insects that work and communicate together in order to ensure the survival of the next generation. The division of labor in the hive is determined by a variety of factors including resource limitation and the number of workers (Hagbery and Nieh, 2012; O'Donnell et al., 2000). Foragers, usually the oldest of the work-force, can travel multiple kilometers in order to find pollen and nectar, and can be specified to one of those tasks (Free, 1955). Most are able to collect both resources in the case that the hive is not bringing in enough pollen or nectar; their behavior is plastic (Hagbery and Nieh, 2012; O'Donnell et al., 2000). The middle-aged workers can act as guards to the entrance and aid in hive maintenance i.e. building up wax around storage pots, chewing pollen and mixing nectar with enzymes to make honey (O'Donnell et al., 2000). The youngest individuals are too soft-bodied to do any intensive labor. Until their bodies dry and harden, they feed on the nectar and pollen. Though *Bombus* species exhibit weak age polyethism (performing different jobs at different ages), they are plastic in their behaviors and can revert to past behaviors (O'Donnell et al., 2000).

Bombus terrestris, also known as the buff-tailed bumblebee, humble-bee or large earth humble-bee is named for the coloration on the queen's abdomen. They are black with a yellow band in front of the wings and across the abdomen. Although it can be difficult to distinguish between workers and males because of their similar size, the males have a distinctive yellow tuft of hair on their nose. The queen is easy to identify because

she is significantly larger (sometimes over 2.5 to 3cm). *Bombus terrestris* is found in Europe, North Africa, and in both west and central Asia. They pollinate a variety of plants, including *Solanums*, fruit trees, raspberries and blueberries (Campbell and Jacinto, 1758). In the U.S. alone, bee pollinated crops were worth $20 billion in 2000. This is equivalent to one out of every three bites of food (Brodie, 2013). In Europe, 84% of crops are pollinated by bees; 1/3 of those are pollinated by honey bees and 2/3 by bumblebees (Brodie, 2013).

Bumblebees, unlike their widely used commercial cousin, the honey bee (*Apis mellifera*), are not well studied. Their colony life cycle is brief, lasting only from the spring to fall, with the only surviving members being the fertile gynes (Free, 1955). This does not allow for easy commercial-pollination because the hives have to be built up every spring, which is time-dependent on temperature and resource availability (Free, 1955). The nest is highly disorganized with pots of "bee-bread" (masticated pollen mixed with enzymes) interspersed with nectar and brood pots. In contrast, honey bees are very organized with whole frames being devoted to pollen, brood, or honey storage.

The way in which honeybee and bumblebees behave is also different. Bumblebee communication is contact-dependent, unlike honey bees which are pheromone-dependent (Dornhaus and Chittka, 2004.). Bumblebees do not actively participate in trophallaxis (the transfer of nectar from the honey-stomach of one bee to another via the proboscis), and are not very susceptible to proboscis extension reflex assays; an assay used to determine learning and memory length (Dornhaus and Chittka, 2004.). The famous

"waggle-dance" of honey bees is also not a behavior displayed in *B. terrestris.* Similarly, honey bees will forage in a flower-specific manner (from the same species of flowers) until the resources are depleted, while bumblebees do not forage using this approach. Bumblebees are more cost-efficient in terms of energy, preferring to travel in straight lines, leaving patches before the resources are completely farmed out (Orban et al., 2012; Rasheed and Harder, 1997). Interestingly, the bumblebee is one of the only organisms that can pollinate the *Solanum* family: potatoes, tomatoes, nightshade, etc. The tubular anthers of *Solanum* hold pollen tightly and it is released using buzz-pollination. The bee will hold onto the anthers and vibrate its flight muscles to a specific frequency thereby shaking the pollen loose (King and Buchmann, 1996). Most pollinating insects, including honey bees, do not have enough body mass to accomplish this, making bumblebees one of the most important insects in pollination.

Bumblebee Olfaction

Bumbles smell via the antennal pathway. Sensilla trichodea on the flagellum of the antenna are stimulated by scents that excite the neurons. Charge accumulation via excitation creates a gradient inside the neural cells, and when the action potential threshold is crossed, an electrical impulse is sent to the glomeruli in the mushroom body of the bee brain where it is recognized as either attractive or repulsive (Benton, 2006). Sensilla trichodea are grouped by a and b types. In *B. terrestris*, there are more type a sensilla on the right antenna, and larger bees have higher numbers and densities of both types. This means that larger bees are more sensitive to lower concentrations of odors; it

takes less of the scent in order to create an action potential. Electroantennography, the measurement of the action potentials in the antennae after stimulation, has revealed that there are no differences in singular antennal odor detection, but when a proboscis extension reflex assay (PER) is performed, disparities between antennal sensitivity arise (Anfora et al., 2011). Using PER to assess learning, Anfora et al. (2011) found that a correct response (an extension of the proboscis, which exhibits learning) to sucrose exposure was 70% when both antennas are used in training. However, when only one antenna is used to train the bee, the left antenna responded correctly 7% of the time while the right is correct 60%. Therefore, short term recall memory is right-side dominant in bumblebees (Anfora et al., 2011).

In general, larger insects have higher electroantennogram responses to similar odor concentrations due to more sensilla trichodea and pore plate sensilla (Spaethe et al., 2007). However, this does not correlate with learning performance; larger bees perform comparably to smaller bees when exposed to the same concentration of scent in a Y-maze behavioral assay (Spaethe et al., 2007). However, larger bees respond more correctly to lower odor concentrations, which allows for bees greater in size to be more efficient foragers, thereby increasing the division of labor in hives (Spaethe et al., 2007).

Flower Choice and Pollination

Which plant a pollinator chooses to visit is not solely defined by initial attraction. Learning also affects visitation rates in bumblebees. They can remember the positions of nectar-rich shoots and visit them significantly more than nectar-poor shoots (Thomson,

1988). The bees will continue to visit the nectar rich shoots even if the nectar is removed from them mechanically after the visitation. The sex of an umbrel and the number of flowers on a plant can also affect alighting. Bumblebees prefer the male phase umbrels more strongly if they have previously fed on other male-phase umbrels (Thomson, 1988).

Honeybees can also develop bias in flower choice though learning. If a honey bee is conditionally trained by receiving high quality nectar when being exposed to a scent, the bee is significantly more likely to visit a flower with the same odor. Training using poor quality nectar and a scent results in the bee avoiding that scent (Wright et al., 2009). Therefore, olfactory bias can be obtained by changing the quality of nectar rewards during associative training; if there is a greater discrepancy in one nectar reward relative to the other, the bee will have a greater bias toward that scent. Extrapolating from this, nectar composition differences in flowers can influence odor recognition by pollinators, which in turn can influence the evolution of floral scents (Wright et al., 2009).

Another factor affecting flower choice in bees is the division of labor in a colony. On average, 16-36% of bumblebee foragers collect either pollen or nectar, the remaining percent of adult foragers are generalists (collect both resources). The first flight out to forage determines which resource the bee will collect for the rest of its life. However, the generalists are able to bring in either resource more frequently if the hive is in need (Hagbery and Nieh, 2012). Interestingly, pollen collectors will make on average 1.61-1.67 times as many trips as nectar specialists, and all foragers are significantly larger than bees limited to the hive (Hagbery and Nieh, 2012).

Honey bees behave similarly, with specialists choosing flowers based on what resource they are foraging for. For example, female flowers have twice the nectar as male flowers in lavender (*Lavadula stoechas*). In field studies, the time spent on flowers is proportionate to the number of female flowers while inflorescence size is inversely correlated with female flower number (Gonzalez et al., 1995). This is hypothesized to be a morphological cue for bees, as the foragers choose inflorescences with a greater female or male flower number (relatively) to suit their needs (Gonzalez et al., 1995). Pollen also influences which flowers a honey bee is attracted to. In an environmental flight cage containing feeders and bowls of pollen, pollen analogs and pollen lipids, there are no significant differences in feeder choice (Pernal and Currie, 2002). However, honey bees prefer particle sizes of pollen below 150µm with the greatest attraction for particle sizes below 45µm (Pernal and Currie, 2002). Protein content is not assessed by the foragers, but handling time is. The amount of time it takes to reach and extract the pollen (via constricting wire tunnels) is inversely correlated with visitation rate. Odor is also assessed by pollinators to determine flower choice, and pollination. When an odor is presented simultaneously with other stimuli and pollen, odor is the primary cue that influences the honeybee's flower decision (Pernal and Currie, 2002). Similarly, bumblebees (*B. impatiens*) can discriminate between rewarding and unrewarding artificial flowers that vary slightly in hue if presented with a paired scent during conditioning (Leonard et al., 2011). If a bumblebee is first conditioned with the color of the flower and pollen (no scent) the degree of uncertainty (choosing a flower that is of

slightly different color than the one trained on) is significantly higher, than those trained with the scent, flower color and pollen (Leonard et al., 2011).

Various species of insects other than bees maintain similar learning capabilities. Ants can associate sucrose and odor during foraging cycles to locate nectar rich plants. Trophallaxis of a scented solution results in Y-maze behavioral tests that indicate that ants can recognize the scent of the solution (between 2 possible scents) and choose the arm containing the scent significantly more often than the non-associated scent (Provecho and Josens, 2009). The opening of maxillary palps in desert locusts (*Schistocerca gregaria*) also acts as a measure of learning. The palp's diameter in response to a specific scent after paired conditioning sessions of odor and food reward is much larger than the response to the odor following unpaired presentations of just food or scent. This memory can last for 24 hours as demonstrated through Y-maze assays of trained and naïve locusts (Simoes et al., 2011).

Attraction to flowers has many components. In field experiments, the number of umbrels per inflorescence, flower per umbrel, and the amount of pollen and nectar per flower can influence a bumblebee's attraction to a plant (Thomson, 1988). Hymenopterans also use visual cues such as the blue color of flowers, flower size, shape and position of nectar guides to locate plants (Burger et al., 2010). In a laboratory setting (artificial flowers), bumblebees are especially attracted to plants with blue flowers and radial nectar guides (Hudon and Plowright, 2011). Volatiles also play an important role in attraction. A plant's volatile profile is used by some bees as a cue to discriminate from

host and non-host plants (where they gather resources) and can cause alighting (landing) onto flowers (Burger et al., 2010). Olfactory cues are essential for oligolectic bees because it allows the insects to discriminate between plants from short distances. Visual cues are less important for oligolectic bees relative to olfactory cues, but when both cues are present, they synergize to increase the discrimination ability of the bees (Burger et al., 2010). Remarkably, bumblebees do not evaluate plant species only on intrafloral characteristics, but also on protein content of pollen (unlike honeybees), total protein per plant, foraging costs per plant, and site-specific efficiency of protein harvesting (per patch). This allows for proper larval development and maximizes forager lifespan via less energy exerted per foraging trip (Rasheed and Harder, 1997). Foragers can also learn to associate forager-deposited odor marks with high quality resources. In an array of eight feeders, a bee will be more attracted to a rewarding feeder even if its position has been moved after the initial foraging trip and the resource removed (Renner and Nieh, 2008). Activated foragers (foragers contacted by bees returning from a collection flight) do not significantly prefer the feeder that the activating bee has returned from; they may prefer their own scent marking at a high-quality resource. However, the bees that are activated are significantly more likely to leave the nest to forage (38.6% increase in foraging attempts on empty feeders) than bees that are not contacted; successful foragers can incite resource collection behavior in other bumblebees (Renner and Nieh, 2008).

Flower choice, pollination rates, and the number of foraging flights, are limited by how far a flower is from the colony. Feeder locations do not affect colony activity level (always active) or pollen size load, but feeders that are far from the hive decrease the

number of flights that the bees are able to undergo (Orban et al., 2012). A mathematical way to understand this is by a simple equation: E (energy) = E-out (flight energy and energy used during resource collection) – E-in (energy gained from nectar/honey). Bees maximize energy (leading to longer lifespans and therefore a healthier colony via increased resource collection) by decreasing flight time as the insect will use the same amount of energy to collect the resources each time (full load of pollen or nectar). Therefore, a plant that is much closer but lower in resources is more likely to be visited than a far-away plant that is resource rich.

The ability to correctly distinguish between newer and older flowers positively affects a bee's energy reserves, and therefore, flower choice. *Bombus pullatus*, *Euglossa erythrochlora* (Orchid Bee), and *Pseudoaugochloropsis graminea* all forage more frequently at new flowers (opened that day) than older flowers that opened prior to the day of foraging; the newer flowers are more likely to have full pollen loads which facilitates pollen collection (Shelly and Villalobos, 1997). This behavior maximizes efficiency because fewer flowers have to be visited in order to fill the corbicula of the bee. From 9-11 am, however, both old and new flowers are visited at the same rate; the old flowers are not depleted of pollen and visiting them will not exhaust the energy reserves of the bee by flying to empty flowers. *Euglossa erythrochlora* and *P. graminea* spend more time harvesting pollen during the first and second visits to a new flower than initial visits to an old flower. Primary visits to new flowers are also greater in length than final (seventh or later) visits to a new flower (Shelly and Villalobos, 1997). This is due to

the amount of pollen left in the flower, and the bee maximizing energy per foraging flight.

One of the most interesting aspects of bumblebees is their ability to buzz-pollinate, also known as sonication. This process influences flower choice because a bumblebee's greater body mass makes it one of the only species that can tap the pollen rich plants of *Solanum*. Plants that are buzz-pollinated generally have radially symmetrical flowers that are pendant in form; they are isolated from the leaves of the plant by a long, flexible pedicle. The petals of the flower are reflexed to allow the pollinator to hang onto the cone of stamens, which have robust filaments and poricidal anthers. A simple style protrudes from the anthers, and the plant does not produce nectar (Harder and Barclay, 1994). At a certain frequency, a sonic release of pollen occurs (King and Buchmann, 1996). The release is caused by centrifugal forces created by the bee's vibration, pulling the pollen out of the apical pores in the stamens. In *S. laciniatum* (kangaroo apple or poroporo) the fundamental frequency of the stamen is 124 Hz. Bumblebees vibrate at 180 Hz which can release 72,000 grains of pollen at a time, only 18% of the total grains (King and Buchmann, 1996). As the bee increases its wing-beat frequency it generates high acceleration forces, releasing the pollen from the anthers via thoracic vibrations equivalent to "the maximum possible displacement of the indirect flight muscles (King and Buchmann, 1996)." The relatively low release of pollen in the poroporo is a product of "the gradual dehydration of the tapetal fluid remaining in the lower regions of dehiscent poricidal anthers produces a timed release mechanism (King and Buchmann, 1996)." This mechanism meters out pollen in order to attract multiple

pollinators, increasing the number of other plants receiving its germ cells, thereby increasing the plant's fitness.

Pollinators of *Solanum*

There are numerous insects that pollinate *Solanum* plants due to distribution, climate range, size and evolution (plants that evolved a volatile profile to fit their preferred pollinator). The *Solanum's* fertilization requires buzz pollination, and because tomatoes and tomatoes are cash crops, there has been a push to commercially pollinate greenhouse grown crops. Therefore, it is beneficial to identify which pollinators are specific to which species of *Solanum* (plant preference) as well as if one species of insect can pollinate multiple crops.

Tomatoes in Central Europe are pollinated mainly pollinated by *B. pascuorum* (brown bumblebee) and *B. terrestris. Bombus lapidarius* (red-tailed bumblebee) and *Megachile willughbiella* (Willughby's leaf cutter bee) females also known to pollinate the plants, but *B. sylvarum* (shrill carter bee) rarely does (Teppner, 2005). A tomato anthesis (the period of time when a particular flower is reflexed and functional) is open for 2-3 days with the anther open from the morning until the afternoon of the first day. Smaller bees (4-6mm) such as *Hylaeus gibbus* females and *Lasioglossum morio* and *L. polito* appear to buzz-pollinate (inferred) but afterwards scrape off the pollen stuck to the anthers following vibration as their main means of pollen collection (Teppner, 2005). Australia does not have native bumblebees and is exploring means of pollinating tomatoes in commercial greenhouses. Bringing non-native organisms into a new habitat

has unforeseen consequences and the recently introduced *B. terrestris* in Tasmania presents a threat to native ecosystems as an invasive species (Hogendoorn, 2000). Therefore, Australia is looking into its native carpenter bee (*Xylocopa lestis*) as a substitute. One study by Hogendoorn in 2000 highlights the efficacy of carpenter bee pollination. He discovered that greenhouse grown tomatoes that are pollinated by female carpenter bees have more seeds and heavier fruits than tomato plants that are not pollinated (Hogendoorn, 2000).

The push for commercial *Solanum* crops has created the need for pollination in many countries, including Mexico, leading to multiple pollination studies. One of the first such studies by Pressman and others (1999) compared bumblebee pollination to an "electric bee," a type of mechanical pollination. During autumn and early winter, no difference in fruit set, seed number, fruit size and overall yield occurred (Pressman et al., 1999). During severe winter conditions, however, bumblebees were more efficient than using an electric bee two to three times a week; low amounts of pollen per flower still resulted in a large fruit set. However, if the electric bee is used daily under harsh climate conditions, the same fruit set is achieved. Pressman's team concludes that bumblebees are efficient pollinators when flower organs are viable and there is a certain amount of pollen available. They also maintain that to achieve the same efficiency year round as bumblebees, the frequency of mechanical pollination should be flexible, with more operation under winter conditions (Pressman et al., 1999). This begs the question if mechanical vibration is economical or even possible within large commercial greenhouses. However, these results contradict future findings. For example,

Nannotrigona periloampoides pollination was measured for foraging attempts, percentage fruit set, seed set, weight of fruit and overall fruit production per square meter and compared with mechanical vibration and no pollination in the tomato *Lycopersicum esculentum* (Cauich et al., 2004). Trials using stingless bees produced more fruit than those performed with no pollination, but the fruit weight was not significantly different. Mechanical vibration produced the highest seed set with the number of seeds correlating with fruit size (Cauich et al., 2004). Although stingless bees could be an alternative to the local Africanized *Apis mellifera* and non-native *Bombus* species in Mexico, more research is needed to evaluate the cost/benefit ratio on large-scale greenhouses. The stingless bee's populations declined throughout the 2 months of testing and did not create large tomatoes with high seed numbers (Cauich et al., 2004). Similar studies are being undertaken in Europe. They are experimenting with flower vibration, bumblebee pollination and auxin spraying to compare fruit production in greenhouses. Environmental constraints limit pollination in early and late seasons, which may warrant the use of different pollination methods to increase overall production and decrease costs (Martin-Closas et al., 2009). However, bumblebees are the best pollinators, with the most fruit produced, while auxin and mechanical pollination are effective in the first crop but not the second; there are two in every year. Bumblebees are the most effective after cool nights in the early cycle because the fruit set is limited due to lessened production of viable pollen and smaller pollen releases (Martin-Closas et al., 2009).

Honey bees (*A. mellifera*) have also been proposed as a pollinator of greenhouse tomatoes when no other bee is readily available. To test this idea, colonies were set up in

the winter and screened into the greenhouse in the summer to promote tomato foraging (Sabara et al., 2004). Hives were set up with either two brood frames or two empty frames, but this did not result in fruit quality differences between colonies. Fruits that were pollinated by honey bees (via landing on the anthers and picking up loose pollen) did have significantly more seeds than bagged flowers (no pollination). A 98% fruit set was achieved for both test colony types compared to the bagged plant's 80% fruit set. However, summer fruit did not differ significantly in seed number for the bagged and pollinated plants (Sabra et al., 2004). Therefore, the time of year significantly affected the quality of fruit pollinated by *A. mellifera* because seed numbers observed between the treatments after mid-April were similar to the bagged plant's fruit seed set. Overall, managing brood levels and vent screening did not improve the quality of fruit pollinated from honey bees. However, Sabra and others (2004) maintain that honeybees can be a viable alternative for pollinating greenhouse tomatoes, but only during the winter. A previous study by Sabra and Winston also highlights this point. Hives that are placed in greenhouses during the winter reared brood that was maintained at low levels in colonies with or without added brood after 21 days. The bees that did emerge (from winter and summer; winter has screened greenhouses while summer has both screened and unscreened) were significantly lighter than bees from outdoor colonies (Sabra and Winston, 2003). Honey bee flight activity was not affected by initial brood level but during the summer the screened colonies had brood production nearing zero after twenty one days. The unscreened greenhouses had higher levels of brood production due to the ability to forage outside and gain a diverse diet in terms of protein (pollen from different

plants). Screening did not affect flower visitation which means that in order for honey bees to be an economically viable option for greenhouse tomato pollination the owner must replace the hives every 3 weeks (Sabra and Winston, 2003).

Although the main focus of this review is on tomato pollination it is also useful to look at the pollination of another *Solanum*, eggplants (*Solanum melongena*). Like the tomato, there is a reduced seed set in the absence of pollinators, and seed numbers increase when pollen deposition is enhanced. In Kenya, *Xylocopa caffra* and *Macronomia rufipes* are effective pollinators of field eggplants, but the visitation rates of the bees decline significantly with increased distance from the wild habitat of the bees (Gemmill-Herren and Ochieng, 2008). As with all bees, protein diversification is necessary for proper brood development, therefore, the areas surrounding crop fields are an important tool for assessing visitation rates. In Kenya, farm weeds provide the main pollen resources, but the *Acacia tortilis*, the umbrella thorn acacia river forests, experience high visitation rates during the dry season. Therefore, this temporal diversification in resources highlights the need for conserving natural ecosystems and interspersing wilderness with crop lands in order to increase pollination (Gemmill-Herren and Ochieng, 2008). Another study assessed which pollinators are found on eggplants, and their pollination efficiency. No pollination results in a failure to form fruits, and if fruits do form they are much smaller and lighter than insect pollinated and pollen supplemented plants (Patricio et al., 2012). Plants that are hand pollinated, or supplemented, have similar fruit weights, but insect pollinated plants have varying weights that differed significantly (may be pesticide related interference). The buzz-

pollinators captured on the plants were species of *Bombus, Xylocopa, Exomalopsis, Centris, Oxaea* and *Halictidae*. *Trigona* sp. and *Apis mellifera* were also collected on the flowers (Patricio et al., 2012).

Greenhouse Colony Activity and Health

Bumblebees are the main pollinators of greenhouse tomatoes. Therefore, measuring the health of the colony activity is essential for ensuring high pollination levels. One way of determining pollination levels is to check the flowers for bruising. After a bee visits a flower and sonicates, the flower becomes duller (bruised), and with subsequent visits becomes even darker in color. Another way to measure colony activity is to implant a photodiode monitor into the hive entrance to measure the bee trips per hectare of land. Levels of pollination positively correlate with bee activity level up to a mean of 400 pollen grains harvested per stigma per day at 2000 total trips per hectare (an average of 4.87 trips per bee per day). Pollination decreases with distance from the hive down to 200 pollen grains per day at 50m from the hive (Morandin et al., 2001). Any further activity does not result in an increase in pollen harvest. To maintain this level of pollination, 7.6 to 19.8 colonies must be present in each hectare of land, with an average of 11.6 (Morandin et al., 2001). Climate, hive size and distance between plants affects colony activity levels (trips per bee per day), which accounts for the large variation in the number of colonies necessary per hectare of land in order to ensure high levels of pollination (Morandin et al., 2001). If hives are not pollinating at a high level, an increase

in ambient temperature will create greater hive activity, and evenly distributing the hives will create fewer distal zones of low pollination (Morandin et al., 2001).

Greenhouses pose resource constraints that are detrimental to *Bombus* colony health. The singular pollen source leads to smaller brood size (weight), leading to decreased foraging throughout the year. They will leave the greenhouse to forage on other plants if possible. Competition for resources can also be a detriment to colony health. There is evidence that the presence of multiple species of *Bombus* (specifically *B. occidentalis*, the western bumblebee and *B. impatiens*, the common eastern bumblebee) residing in the same greenhouse can impede colony growth (Whittington and Winston, 2004). *Bombus impatiens* rear more brood and workers while also foraging more often (trips per hour) than *B. occidentalis*. However, they also returned to the colony without pollen loads and left the colonies without depositing the pollen more frequently than *B. occidentalis.* From this observation the authors concluded that *B. impatiens* is a less effective forager in greenhouses (Whittington and Winston, 2004). However, the presence of *B. impatiens* has a deleterious effect on the *B. occidentalis* populations. *Bombus occidentalis* colonies never reach their full potential in greenhouses. They have smaller hive numbers than colonies that are placed outside in the natural environment, as well as those physically enclosed, protecting them from external conditions (Whittington and Winston, 2004). The species that are not normally in contact in an enclosed environment have unforeseen effects on each other. Therefore, when pollinating a greenhouse, only one species of *Bombus* should be used.

Modifications of Plant Volatile Profiles

Pollen odors are more pronounced in insect pollinated plants than those that are bird or wind pollinated; birds have a very poor sense of smell and use sight to find appropriate food items. Wind-pollinated plants tend to have more alpha-methyl alcohols and ketones, which may serve as a defense against insects; it is more beneficial to the plant to repel insects because they are not needed for pollination. Insect pollinated plants emit various chemicals that serve as a repellent for non-pollinating insects, but they also must be attractive to their host-pollinators (Dobson and Bergstrom, 2000). In essence, the plant must protect its pollen while also increasing its dispersion via animals. Therefore, it has been suggested that volatile organic compounds (VOCs) evolved to attract pollinators and that insects that feed on pollen perceive its odor and are able to differentiate between pollen types (between species) and the relative amount within each plant (Dobson and Bergstrom, 2000). Therefore, the odor of a plant's pollen influences bee foraging in terms of which flower species it will pollinate and also which particular plant of that species it will visit because flower choice is partially dependent on the amount of available resources.

Volatiles emitted by plants can be altered directly or indirectly by various organisms, thereby increasing or decreasing a pollinator's attraction to the plant. These actions can greatly impact a bee's preference towards that species of plant in the future, which, in turn, affects the plant's fitness. An example of this occurs in both above and below-ground herbivory. Barber and associates (2011) found that cucumbers (*Cucumis*

sativus) that are exposed to above-ground herbivory by the striped cucumber beetle (*Acalymma* vittatum) have reduced leaf damage later in the growing season and are attacked less by root herbivores. However, the affected plants have 35% fewer female flowers. Leaf damage to cucumbers caused no reduction in fruit production, while plants with damaged roots produced 38% less fruit and are on average 25% lighter than control plants (Barber et al., 2011). The damaged plants also interact less with both antagonists and mutualists in the soil (Barber et al., 2012). Leaf herbivory reduces pollinator visitation (29% less probing time), while root herbivory increases probing time by 100% (over control plants). This can be attributed to smaller and fewer flowers as the plants shift energy use from reproductive activities to defense (Barber et al., 2012). Another example, provided by Poveda and others (2003), is in potted mustard (*Sinapis arvensis*). If cabbageworms (*Pieris rapae*) remove 50% of a seedling (first four leaves) leaf tissue, the plant will have reduced height and shoot-mass as well as delayed flowering. This results in smaller, less attractive plants during peak pollination periods (Poveda et al., 2003). However, root herbivory induced by wireworms (*Agriotes sp.*) increases pollinator visitation per plant. Combined leaf and root herbivory causes a decrease in flowering period, fruits per plant and seeds per fruit (Poveda et al., 2003). Although the high amount of herbivory reduces the growth of the plant, reproduction (seed set and weight per plant) is similar in above-ground herbivory, below-ground herbivory, and control plants (Poveda et al., 2003).

Tomatoes (*S. lycopersicum*) can also experience a shift in VOCs that affects pollinator preference. Herbivory causes the primary metabolic goals of the plant to alter

from reproduction and growth to metabolic precursors for defense compounds. There is a shift from keeping resources in the leaves to mobilization of resources into storage tissues which increases a plant's tolerance to leaf damage (Steinbrenner et al., 2011). Twenty four hours after herbivory via *Helicoverpa zea* (a generalist, new world bollworm, that will feed on any plant) and *Manduca sexta* (a specialist, the tobacco hornworm, that is found on plants from the family *Solanaceae*), metabolic changes appear most strongly (magnitude and concentration changes measured by GC-MS) in apex and root tissues rather than undamaged leaflets of damaged leaves, signifying a rapid, whole-plant response (Steinbrenner et al., 2011). Interestingly, *H. zea* alters more metabolites than *M. sexta*, mainly phenolics and precursor amino acids associated with defense, while metabolites associated with carbon and nitrogen transport are produced after herbivory by *M. sexta*. The majority of changes in metabolism are specific to a tissue or herbivore type (Steinbrenner et al., 2011).

Similar to tomatoes, the Peruvian Nightshade (*Solanum peruvianum*), is also detrimentally affected by herbivory. Herbivory decreases pollinator visitation rates through the emission of defensive VOCs (Kessler et al., 2011). Real herbivory, as well as the application of methyl jasmonate on plants (defense VOC), reduces pollinator attraction, and therefore, seed set of the plant. The solitary bees that visit *S. peruvianum* regularly use the VOCs produced after herbivory, or the presence of methyl jasmonate, as cues to avoid the damaged plants (Kessler et al., 2011).

The Role of Volatiles in Communication

There are many VOCs emitted by a plant, some act as attractants while others are defensive in nature. Transmission of signals in plants is carried out by terpenes, phenylpropanoids, benzenoids and other VOCs. Terpenes are essential for communication processes in living organisms and are quickly emitted due to exogenous influences (Pavarini et al., 2012). The amount of each compound being released also fluctuates during the day. Bumblebees prefer to pollinate tomatoes that produce less β-phellandrene and 2-carene, and many strains of tomatoes emit these two compounds differentially during peak bumblebee activity. Therefore, β-phellandrene and 2-carene may be antiherbivory VOCs, and tomato plants reduce their production of these volatiles during peak pollinator activity, facilitating the pollination of the plant (Morse et al., 2012). Herbivore-induced volatiles can also act as plant-plant cues priming defenses in neighboring, undamaged plants, as well as causing extrafloral nectar secretions (Li et al., 2012). In Aspen (*Populus tremula x Populus tremuloides*), plants that are exposed to VOCs released by damaged plants produce EFN sugars, but do not change the amount of terpenes that they emit. However, when those plants previously exposed to VOCs from other damaged plants are exposed to herbivory (3rd in line), they release significantly more terpenes than previously unexposed plants (Li et al., 2012). EFN secretions are not increased in subsequent attacks, meaning that "herbivory-induced VOCs prime VOC emission but not EFN secretion (Li et al., 2012)." However, larvae of the Autumnal moth (*Epirrita autumnata*) reduce the production of EFN sugars systemically, suggesting that the EFN defense is case dependent on plant and herbivore type; different plants respond

in a variety of ways to herbivory-induced VOCs and subsequent herbivory in order to optimize the use of resources (Li et al., 2012).

Plant-plant communication can even cross the species boundary. Transgenic tobacco plants (*Nicotiana tabacum*, NtOS2) that emit E-beta-ocimene prime the defenses in lima beans (*Phaseolus lunatus*) that are infested with spider mites (*Tetranychus urticae*), which are highly induced to release VOCs (Arimura et al., 2012). The volatiles released from the first receiver lima beans also affect a second receiver (lima beans) that are also exposed to spider mites. The homoterpenes [(E)-4,8-dimethyl-1,3,7-nonatriene and (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene] are emitted at higher densities than volatiles released by plants that are placed near infested, WT-volatile-exposed plants (Arimura et al., 2012). These airborne cues can also act on parts of the plant that are not vascularly connected. Hybrid poplar trees (*Populus deltoids* x *nigra*) that are wounded by herbivores prime defenses in adjacent leaves that have no vascular connection to the wounded ones (Frost et al., 2007). Undamaged leaves exposed to the herbivore induced volatiles have greater defense responses to feeding by gypsy moth larvae (*Lymantria dispar*) than leaves that are not previously exposed. These localized signals may encourage a systemic readiness to resist herbivory (Frost et al., 2007).

Herbivory can also lead to a tri-trophic interaction via volatile communication. Green-leaf volatiles (GLV's; terpenes and lipoxygenase pathway products) are used by carnivorous insects to locate prey (Pinto et al., 2007). However, GLVs are reactive with atmospheric pollutants, and greater levels of ozone may affect the tri-trophic system. In

two tri-trophic systems: 1) cabbage-diamondback moth- diamondback moth parasite and, 2) lima bean, red spider mite and persimilis (*Brassica oleracea-Plutella xylostella-Cotesia Plutellae* and *Phaseolus lunatus-tetranychus urticae- Phytoseiulus persimilis*) that are exposed to enhanced ozone levels (60 and 120 nl^{-1}) completely lose all terpene and GLV signaling (Pinto et al., 2007). However, the parasitic predators are not disturbed by the loss; they orientate to other volatiles also produced by herbivory. Benzyl cyanide (nitrile in cabbage) and methyl salicylate (in lima beans) are not reduced when exposed to ozone, suggesting that more stable herbivore-induced compounds can orientate carnivores, and may be used in ozone rich environments (Pinto et al., 2007).

There have been few studies on whether cultivars of the same species produce the same VOC profiles, either in defense or pollinator attraction. In strawberries (*Fragaria x ananassa* Duchesne), GC analysis reveals that although there is no difference in volatiles emitted between strains, the relative quantities of each compound and total amount of volatiles released differs between Sonata, Honeoy and Darselect strains (Klatt et al., 2013). Sonata emits a larger amount of volatiles as well as a higher level of most individual compounds that elicit an electroantennographic response in red mason bees (*Osmia bicornis*). Sonata is also the most attractive variety, accruing significantly more pollinator visits under field conditions (Klatt et al., 2013). The differences in the amounts of emitted compounds among varieties mediate pollinator attraction in *O. bicornis* and because fruit quality and number depend on pollination, more attention should be paid to what varieties produce what VOCs, and how crossing certain strains could affect the volatile profile of the following generation. For example, some strains of tomatoes (*S.*

lycopersicum) overexpress the Prosystemin gene (35S::PS) or have a mutation in the JA (jasmonic acid) synthesis pathway (def1) which reduces their wound response (Degenhardt et al., 2010). Plants that overexpress 35S:PS have greater amounts of emitted volatiles than controls and increased accumulation of proteinase inhibitors. JA application to tomatoes shifts the metabolism of the plant to increased defense protein accumulation and volatile emissions in wild-type plants, but does not increase volatile emissions of 35S::PS plants (Degenhardt et al., 2010). If a tomato is wounded by *M. sexta*, the wild-type plants increase defense protein synthesis and volatile emissions, but def1 plants do not. Def1 plants that receive a JA application restore their local and systemic aggregation of defense protein and herbivore-induced volatile emissions. These findings suggest that prosystemin and JA-signaling carry a role in the emission of volatiles in tomatoes, which are essential to pollinator signaling (Degenhardt et al., 2010).

As previously stated, strains of the same species of plant have varying amounts of volatile compounds and overall volatile production. This raises the question of if herbivory or wounding causes the same defensive volatile emissions in each cultivar. Oriental leafworm moth (*Spodoptera litera*) herbivory and mechanical injury can cause five cultivars of tomatoes to release monoterpenes (65% of emissions), sesquiterpenes (15%) and aldehydes at 10% (Raghava et al., 2010). Subsequent tests using the herbivory-induced volatiles on *Trichogramma chilonsis Ishii*, a stingless wasp that parasitizes insect eggs, reveals a strain-specific olfactory response in terms of attraction (Raghava et al., 2010). The variation in amount and time of release of volatiles specific to each cultivar suggests that the metabolites are genetically determined and can be altered

using artificial selection (Raghava et al., 2010). For example, inbreeding in horsenettle (*Solanum carolinense*) results in plants that emit more volatiles regularly, but when they are exposed to herbivory they have a smaller induction of volatiles (Kariyat et al., 2012). Additionally, compounds related to the recruitment of predators and parasitoids (terpenes) are either emitted in smaller quantities or not at all. Undamaged inbred plants also attract more herbivores and recruit fewer parasites than undamaged or outcrossed plants. Inbreeding, therefore, negatively impacts the volatile profile of a plant, attracting more herbivores and less predatory insects (Kariyat et al., 2012). Inbreeding may be a large problem in contemporary agriculture, as the effects on pollinator attraction relative to cash crops are not widely studied.

Purpose

The purpose of the studies described here was to determine if bumblebees (*Bombus terrestris*) were differentially attracted to artificially selected tomato strains via olfaction in a laboratory environment. Using Brandywine (Bw), Defiant (D) and Mountain Magic (MM) cultivars, the goal was to understand if by using a Y-maze behavioral apparatus, naïve bumblebees are preferential in their choice of volatile organic compound (VOC) profile. The behavioral assay was divided into 4 sections: 1) using 3g of plant material and directly comparing the strains against each other, 2) whole plants used in a similar manner, 3) comparing two plants of the same strain; one plant was mechanically damaged while the other was not, 4) and testing the pollinator response to jasmonic acid, a hormone used to simulate mechanical herbivory. The first two tests

determined which strain bumblebees are most attracted to while the third test determined if bumblebees are differentially attracted to whole plants, as opposed to damaged ones that are simulating herbivory. The final test determined if jasmonic acid (simulated herbivory) is a deterrent to pollinating bumblebees. I hypothesized that bees would be the least attracted to the most derived strain, MM, and that when damaged, bumblebees would not be differentially attracted to the undamaged or damaged plants. Similarly, bees would be the most attracted to the least resistant strain, Bw, and would be more strongly attracted to the undamaged plant rather than the damaged. The Bumblebees were hypothesized to be deterred by the jasmonic acid, concurring with the damaged versus undamaged plant comparisons.

The rationale for these experiments is that agricultural companies create artificially selected strains that are bred for disease resistance and hardiness. They do not take into account that by selecting for these resistances they may also be unintentionally losing, or modifying, attractive traits; in this case the volatile organic profile of each plant. This loss, or modification, of volatiles may decrease pollinator attraction. It was therefore necessary to test whether pollinators are differentially attracted to plants that are bred for disease resistance or heirlooms, which have similar traits throughout all generations.

Materials and Methods

Tomatoes and Bees

Tomato seeds (*Solanum lycopersicum*) of three varieties, Defiant, Mountain Magic and Brandywine were ordered from johnnyseeds.com in February of 2013. The choice in varieties was made in order to establish a disease resistance gradient of high, medium and no resistance (Table 1). Twenty seeds from each variety were planted in trays and transplanted over the subsequent months to facilitate growth using natural compost and fertilizer. Five Bumblebee hives were bought from Natupol® throughout the summer and fall of the same year to ensure sufficient foragers were available for Y-maze behavioral testing. The bees were allowed to forage freely outside and the hives were left unmolested during testing. Cuttings of the tomato plants were taken in August to start a new line of smaller plants, making them easier to manage in the Y-maze apparatus.

Table 1: List of the three cultivars used in the experiments. Mountain Magic is the most resistant to disease followed by Defiant and Brandywine, which has no resistances. Defiant lacks resistance to all races of Fusarium wilt in comparison to Mountain Magic. Ab is an abbreviation for Alternaria blight, F2 is Fusarium wilt races one and two, V is Verticillium wilt and F3 is the resistance to all races of Fusarium wilt.

	Resistance					
Tomato Strain	ab	f2	f1	f3	lb	v
Defiant PhR (F1)	x	x	x		x	x
Brandywine						
Mountain Magic (F1)	x	x	x	x	x	x

Y-maze

Acrylic Y-mazes were created in the Project Development Lab at Bucknell University by Tim Baker. The single arm was 3" in length while the two bifurcating arms were 5" inches in length, based on parameters in a previous study by Suchet and associatese (2010). The diameter of the square-cut mazes was 1 inch (Figure 2). Caps at the end of the mazes were held on by metal screws with 1/8" tapered holes cut into the plastic to hold 1/8" socket connectors. Black 6mm outside diameter, 1mm wall, linear low-density polyethylene (LLDPE) tubing (NSF 61 certified for use with potable water) was connected to 1.5L cells composed of bisected, FDA approved, soda bottles thoroughly cleaned with 95% ethanol. The caps of the soda bottles also had 1/8" tapered threads to connect to 2L bottles implanted with a 12V, DC, Nidec Model Gamma29 blower fan (0.13 Amp). The flow rate at the end of the 1.5L holding cells was 1500cc/min. The holding cells were swapped out with a more malleable container when whole plants were used. Two Glad® Turkey Oven bags were cut and overlapped with Gorilla Tape® to allow for plants over four feet in height. The top of the bags were connected to three gallon Ziploc® bag tops (to ensure an airtight closing of the bag without having to re-tape every trial). The new holding cells were connected to the 2L and 1.5L bottles via cutting Glad® vacuum bags and rolling the plastic into a tube and inserting the tube 5" from the top and 1' from the bottom of the 4' bag (on opposite sides). The air flowed from the 2L bottles to the holding bags (over the plants) emptying into the 1.5L holding cells and finally ending in the Y-maze where it blows over the insect, allowing two different volatiles to be compared for behavioral preference (Figures

1 and 2). Initially, 3g cuttings (with at least four reflexed flowers) were used in comparing strains. Later, full plants were used in the strain comparison as well. Damaged and undamaged plants of the same strain were also compared after mechanical herbivory (2 hole punches) in 75% of the leaves (Table 2). Only the cuttings and jasmonic acid were placed in the 1.5L holding cells. The full plants were placed inside the bag-containers.

Procedure

Seven bumblebees were captured in the morning (10 am) from varying hives during the week and placed in 20ml scintillation vials with holes punched into the caps. The bees were deprived of food and stored under red-light (which was also the light source for the behavioral tests) for 2.5 hours in order for their hunger motivation to overcome their escape motivation (Suchet et al., 2010). Testing began after the plant tissue (or whole plant) was placed in the apparatus and air was allowed to run for 5 minutes to ensure that the volatiles were being transferred into the Y-maze. To start the test, a single bee in a scintillation vial was placed up against the opening to the maze. A timer was started when the bee crossed the decision point of the maze (where the arms bifurcate). Data were collected for the time elapsed in each scent-offering arm (cumulatively across all 3 minute trials), and also the bee's first choice of arm. Timing continued for 3 minutes. If the bee stopped, flew in the maze, or was otherwise preoccupied (trying to find sugar by licking the maze) that particular trial did not count because the bee was not exhibiting foraging behavior. To ensure enough runs were

completed, the trial was repeated after the bee was returned to its vial, awaiting its next run as the last in line in order to stimulate foraging behavior. Time spent in the single arm or the vial was also counted, but only to ensure that the full 180 seconds were accounted for after the run. The seconds spent in the vial or in the single arm were interpreted as a non-searching act, and therefore, not used to determine the proportion of time spent in search of food between the two present scents. After each bee completed a trial, the maze was disconnected from the tubing and cleaned with water and a brush (acrylic will crack when it comes in contact with ethanol) to ensure no scent marks were left to bias the other bees. Five mazes were used so that the previous mazes had time to dry between trials. After each bee completed the run once, the tubes from each holding cell were switched (opposite arm of the maze) and each bee completed the trial again (total of 14 trials). This was done to ensure that there was no side-bias in arm choice. After the 14 trials, the bees were fed, painted on their thorax using a painter's pen® (to ensure that they would not complete another trial) and released. The plants were also switched out so that every day a new plant was used (to erase plant bias and avoid pseudoreplication). The cuttings stage of the project utilized more runs (26-30) because it was unknown whether the apparatus would work for this experiment (fast enough air flow to stimulate the bees, whether the plastic bottles affected behavior etc.), and it was necessary to understand how many trials it would take to achieve significance. The trials were later reduced when it became clear that the cuttings were not attractive to the bumblebees. Therefore, each cutting trial took three days to complete while the other trials took a

single day. The layout of each trial including control experiments and strain comparisons is shown in table 2.

jasmonic acid was obtained from Sigma Aldritch® in April 2014, and the 100µl of hormone was mopped up using a Q-tip. The hormone-drenched Q-tip was placed into a 1.5L holding cell, while another Q-tip was placed into the other holding cell to control for any scent given off. Ten Bumblebees from two new colonies (also obtained April 2014) were used to determine if the bees had an innate preference or aversion to jasmonic acid in a Y-maze setting.

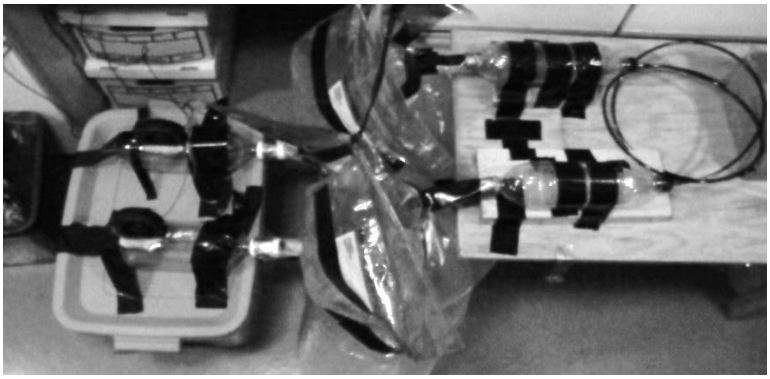

Figure 1: Olfactory apparatus consisting of 2L bottles imbedded with fans that are connected to holding bags, which, in turn, are connected to the 1.5L liter holding bottles for plant tissue.

Figure 2: Acrylic Y-maze which connects to the 1.5L holding bottles via 1/8th inch tapered tubing.

Study Design

Table 2: The experimental design including all 4 test types; cutting strain comparisons, full plant strain comparisons, damaged strains compared to undamaged and a jasmonic acid test. MM is an abbreviation for the Mountain Magic Strain, D is Defiant, Bw is Brandywine, Bl is blank air, Dam represents damaged plants, U represents undamaged plants and JA is jasmonic Acid.

Tissue Type	**Test Type**
Cuttings (1)	
	MM/Bl
	D/Bl
	Bw/Bl
	MM/D
	MM/Bw
	Bw/D
Full Plant (2)	
	MM/Bl
	D/Bl
	Bw/Bl
	MM/D
	MM/Bw
	Bw/D
Damaged Vs. Undamaged (3)	
	Dam-Bw/U-Bw
	Dam-D/U-D
	Dam-MM/U-MM
Jasmonic Acid Test (4)	
	JA/Bl

Methods for Headspace Analysis and GC

A volatile organic compound (VOC) air sample was taken for each strain in the cutting, full plant and damaged versus undamaged groups for a total of nine samples. A Buck® pump was positioned under a glass pipette that contained Porapack® to capture the volatiles drawn through the filter. The glass pipette was inserted into a 1/4" rubber hose that connected to the 1.5L holding cell. A clamp stand was used to keep the pipette

upright during the 6 hour collection cycle. The masses of the plants were not taken in order to normalize VOC emissions because it was necessary to take the VOC profile of each plant as presented to the bees in the Y-maze.

The trap cartridges were constructed from 150 mm glass Pasteur pipettes, stoppered inside with a plug of glass wool, and then gravity packed with 16 mg $\pm$ 4 mg of Porapak - Q, 50-80 mesh (Supelco, Bellefonte, PA, USA). After packing, the cartridges were washed once with 1.5 mL of dichloromethane and 1.5 mL of hexane/acetone (5:1, v/v), then briefly air dried with a 20 second stream of N_2. The VOCs were sampled at a volumetric rate of 1,500 cc/min for 6 hours using a Buck Libra Plus LP5 personal air sampling pump (A.P.Buck, Orlando, FL, USA). After air sampling, the plant VOCs were extracted from the trap cartridges using multiple washes of hexane/acetone (5:1;v/v) spiked with *n*-octadecane (10ng/µL). A typical extraction was washed with 500 µL thrice. Extracts from each of the nine traps (three of cuttings, three of full plants and three of damaged plants, one for each strain) were combined, and stored at -20º C in a closed vial until GC/MS analysis (typically within 72 hours).

GC/MS Analysis

The sample eluate (1-2µL) was splitlessly injected onto an HP-5MS capillary column (30 mm x 0.250 mm x 0.25 µm), with a helium carrier gas at 1 mL/min. The column was installed in an Agilent 5973N mass selective detector. The column temperature program was as follows: initial temperature was 40º C (5 min), followed by a 6º C/min ramp to 150º C and then an 8º C/min ramp to 280º C. The temperature was then held at 280º C for 10 minutes.

Compound Identification

For compound identification, comparisons of sample mass spectra with reference mass spectra from both NIST '05 spectral library and authentic reference samples were performed, as well as matching retention times to appropriate reference samples. The reference samples used were commercially available.

Using Newton's method, the amount of β-phellandrene in each sample (whole plants, cuttings and damaged plants) was calculated (Weisstein, 2014). The method is completed by multiplying the sample area factor by ten and then multiply the resulting number by the extraction amount (usually 750 micro liters), which provides the total amount, in nanograms, of the GC spike. However, there are 3 molecules that have the same response time, β-phellandrene, limonene (a plasticizer found in the air) and 2-ethyl-1 hexanol, which is used to extract β-phellandrene. Therefore, the percentage of phellandrene in the GC spike was determined by dividing the β-phellandrene factor by the sum of all 3 factors, which is equivalent to the percentage of β-phellandrene in the GC spike. The amount of β-phellandrene in the spike is determined by multiplying the percentage of phellandrene in the spike by the total quantity of molecules to get the amount of β-phellandrene in the GC spike.

Analysis

Using SPSS, a paired student's t test was used to determine the significance between the average amounts of time spent in each arm. A chi squared test was used to determine the significance of first choice data. The GC data were analyzed using Newton's method to determine the amount of each compound in each strain's volatile profile.

Results

Cuttings

The testing and comparison of each strain of tomato started with 3g cuttings of each cultivar, placed into the 1.5L holding cells. When compared to blank air, bumblebees were not attracted to the cuttings of each strain; bees spent 51.3% of time in Brandywine, 44.8% of time in Mountain Magic and 41.6% of time in Defiant (Figure 3). When the strains were compared, bees were significantly more attracted to BW than MM (57.9% of time spent in BW arms, $p=0.049$). However, when presented with volatiles from Brandywine and Defiant, the bumblebees did not prefer either of the cultivars (52.6% of time spent in BW, $p=0.54$; Figure 4; Table 3). The bees also did not prefer the volatiles of either Mountain Magic or Defiant when compared with each other (44.7% time spent in MM, $p=0.39$; Figure 4; Table 3). The first choice of arm (out of total choices) was not significant in control experiments or strain comparisons (Table 4).

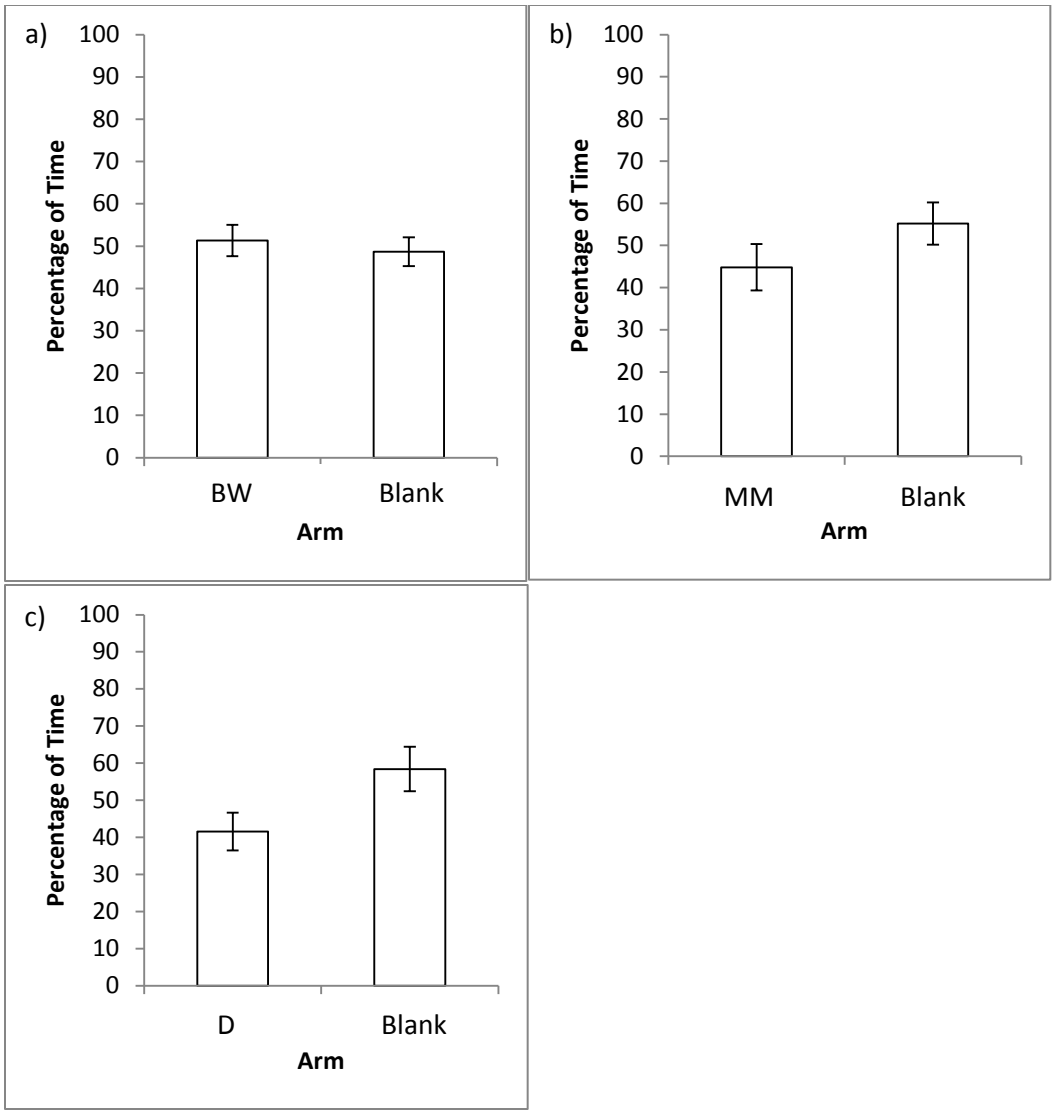

Figure 3: Control experiments (Cuttings): The cumulative time spent in each arm of the Y-maze as a percentage of the total. Part a) refers to the comparison of blank air and Defiant, b) refers to the comparison of blank air and Mountain Magic and c) refers to the comparison of blank air and Brandywine. The error bars represent ± standard error.

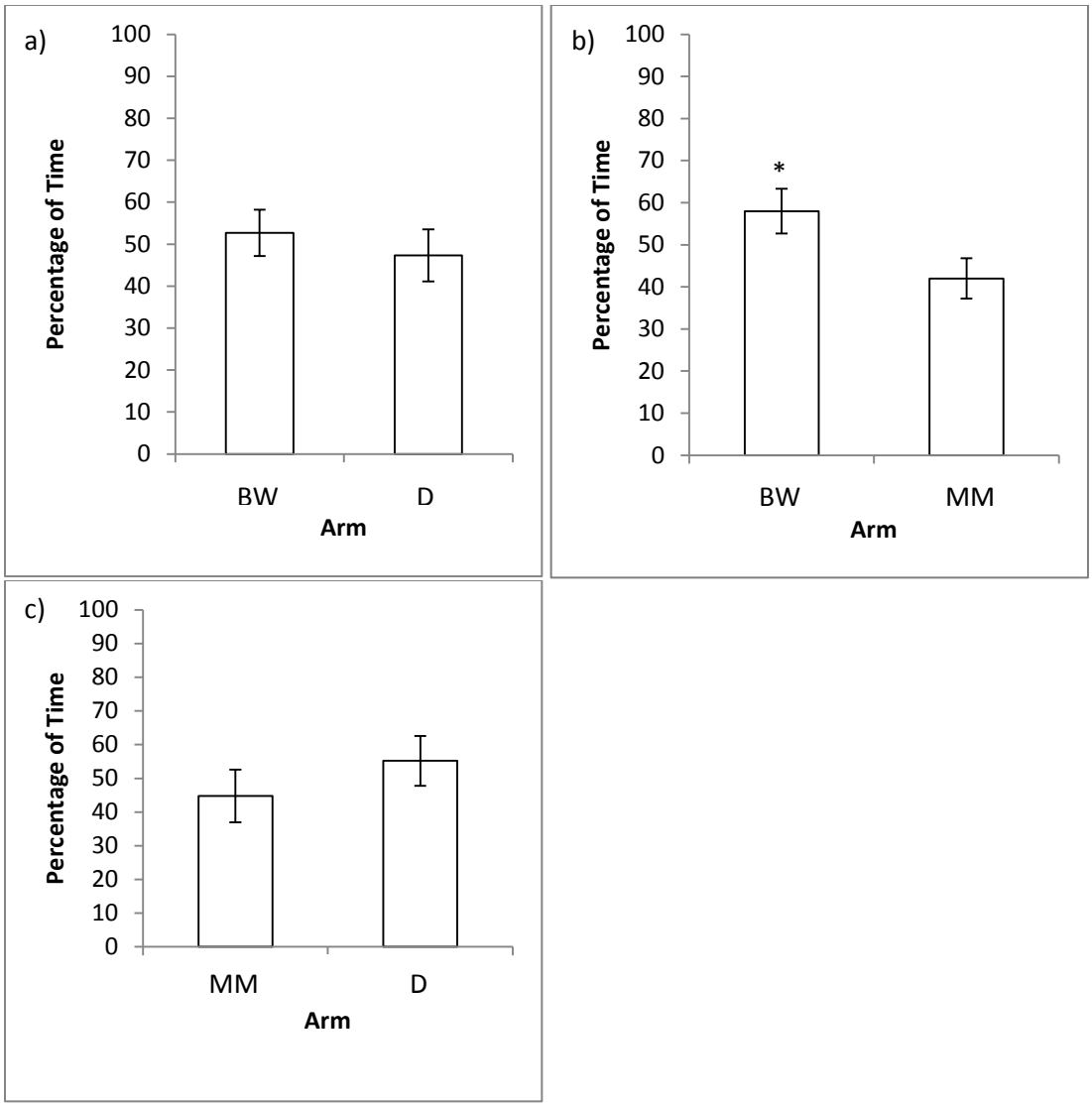

Figure 4: Strain comparisons (Cuttings): The cumulative time spent in each arm of the Y-maze as a percentage of total time. Part a) refers to the comparison of Brandywine and Defiant, b) refers to the comparison of Brandywine Mountain Magic and c) refers to the comparison of Mountain Magic and Defiant. Asterisk denotes significance. The error bars represent ± standard error.

Table 3: Data for bumblebee preference to cuttings of Mountain Magic, Defiant and Brandywine strains when compared to blank air and between cultivars. DF is an abbreviation for degrees of freedom.

Test	N	Total Time in Each Arm (s)	Percentage of Time in Each Arm	Mean (s)	Std. Deviation	Std. Error Mean	T-value	DF	Significance (2 tailed)
Cuttings									
Mountain Magic vs. Blank	28	1208.1, 1485.7	44.8, 55.2	43.1, 53	29.2, 26.2	5.5, 5	1.22	27	0.23
Brandywine vs. Blank	28	1579, 1498.4	51.3, 48.7	56.4, 53.5	19.4, 18.1	3.7, 3.4	0.45	27	0.66
Defiant vs. Blank	26	1103.1, 1549.4	41.6, 58.4	42.4, 60	26.2, 30.7	5.1, 6	1.92	25	0.066
Brandywine vs. Mountain Magic	28	1646.2, 1195	58, 42	58.8, 42.7	28.3, 25.3	5.3, 4.8	2.06	27	0.049
Mountain Magic vs. Defiant	26	1235.5, 1524	44.8, 55.2	47.52, 58.6	39.7, 37.5	7.8, 7.4	0.87	25	0.39
Brandywine vs. Defiant	28	1535, 1380	52.7, 47.3	54.8, 49.3	29, 32.7	5.5, 6.2	0.62	27	0.54

Table 4: Bumblebee first choice data for Mountain Magic, Defiant and Brandywine strains when compared to blank air and between cultivars.

Test	N	First choice	Chi Square	Asymptotic Significance
Cuttings				
Mountain Magic vs. Blank	28	12, 16	0.57	0.45
Brandywine vs. Blank	28	13, 15	0.14	0.71
Defiant vs. Blank	26	12, 14	0.15	0.70
Brandywine vs. Mountain Magic	28	12, 16	0.57	0.45
Mountain Magic vs. Defiant	26	10, 16	1.39	0.24
Brandywine vs. Defiant	28	13, 15	0.14	0.71

Full Plant

Bumblebees did not prefer the volatiles of Brandywine and Defiant when compared to blank air. However, they significantly preferred Mountain Magic over blank air ($p=0.005$) and spent 67% of total food-searching time in MM. For the other two strains, bees spent 63.5% of time in BW, $p=0.071$, and 66.8% of time in Defiant, $p=0.08$ (Figure 5; Table 5). The bumblebees also chose the arms containing the Defiant strain

VOCs significantly more often than the blank arm (11 of 13 trials, 6.2 x^2 value, p=0.013, Table 6). Brandywine and Mountain Magic were not picked more often than blank air containing arms (9 of 13 for BW, 1.9 x^2, p=0.17; 9 of 14 for MM, 1.1 x^2, p=0.29, Table 6). When full plant cultivars were compared, bees preferred BW significantly more than MM; the bees spent 61.5% of time in the BW arm (p=0.015; Figure 6; Table 5). Bumblebees did not prefer either of the arms containing the volatiles of Brandywine or Defiant when compared with each other; the bees spent 60.7% of time in the BW arm (p=0.13; Figure 6; Table 5). Lastly, bumblebees did not prefer the Y-maze arms containing Mountain Magic or Defiant volatiles when compared with each other. They spent 55.1% of time spent in the arms containing MM VOCs (p=0.49; Figure 6; Table 5). The first choice of arm was not significant in full-plant strain comparison tests (Table 6).

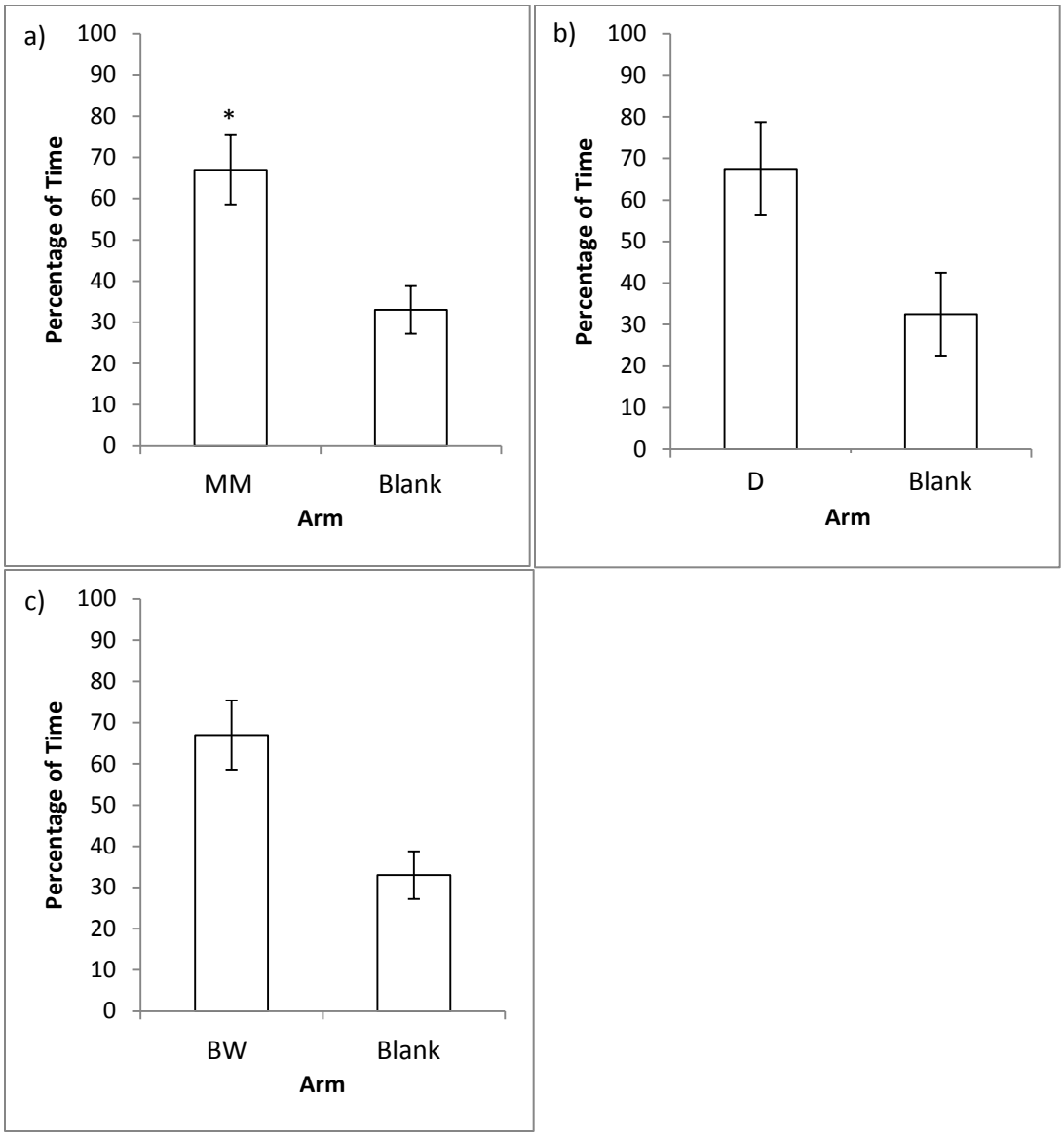

Figure 5: Control experiments (full plants): The cumulative time spent in each arm of the Y-maze as a percentage of total time. Part a) refers to the comparison of blank air and Mountain Magic, b) refers to the comparison of blank air and Defiant and c) refers to the comparison of blank air and Brandywine. Asterisk denotes significance. The error bars represent ± standard error.

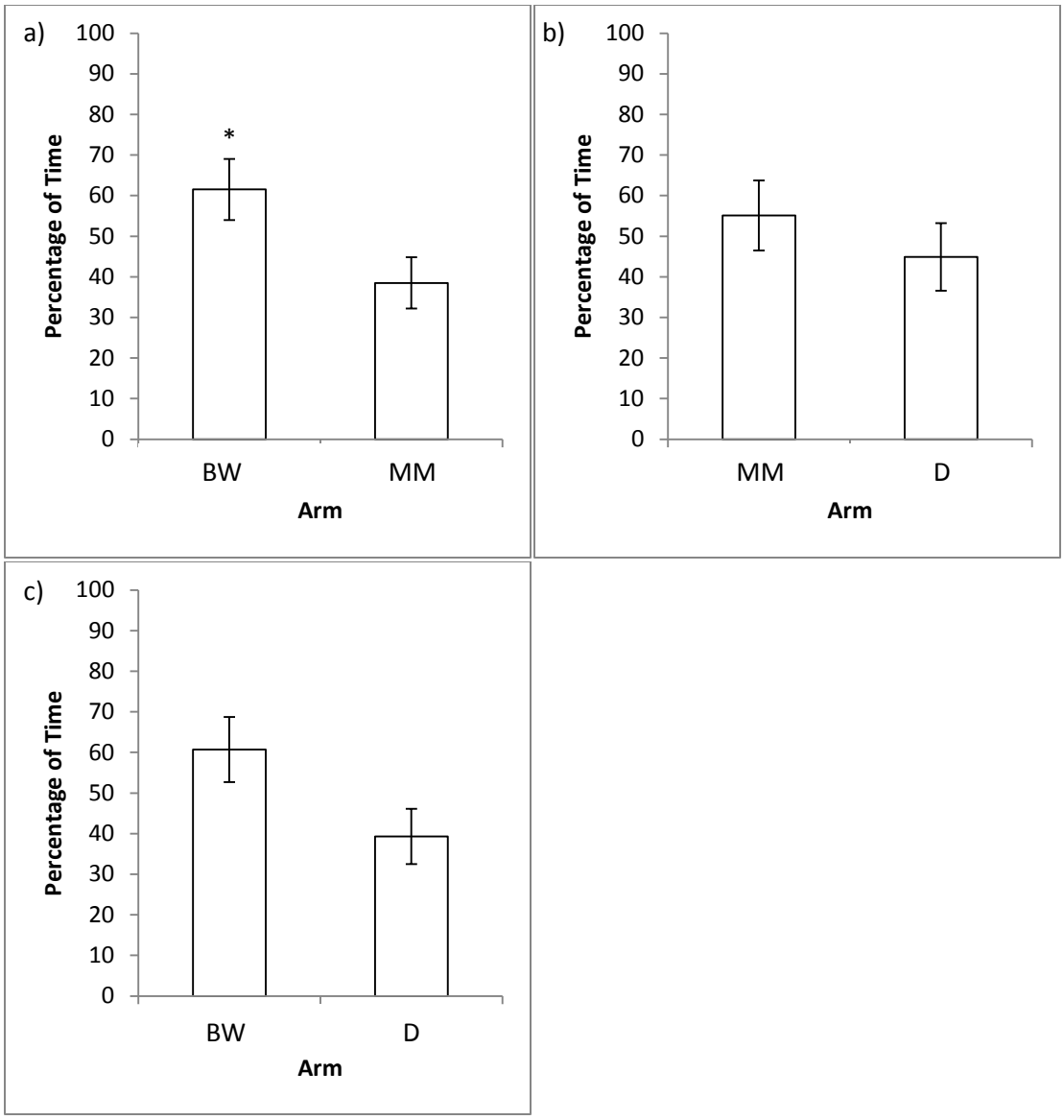

Figure 6: Strain comparisons (Full Plants): The cumulative time spent in each arm of the Y-maze as a percentage of total time. Part a) refers to the comparison of Brandywine and Mountain Magic, b) refers to the comparison of Mountain Magic and Defiant and c) refers to the comparison of Brandywine and Defiant. Asterisk denotes significance. The error bars represent ± standard error.

Table 5: Data for the time that bumblebees spent in the Y-maze arms containing the VOCs of full plants of Mountain Magic, Defiant and Brandywine strains when compared to blank air and each other. DF is an abbreviation for degrees of freedom.

Test	N	Total Time in Each Arm (s)	Percentage of Time in Each Arm	Mean (s)	Std. Deviation	Std. Error Mean	T-value	DF	Significance (2 tailed)
Full Plant									
Defiant vs. Blank	13	886.5, 425.4	67.5, 32.5	68.2, 32.7	40.5, 35.9	11.2, 10	1.92	12	0.08
Brandywine vs. Blank	13	920.8, 528.3	63.5, 36.4	70.8, 40.6	36.5, 28.2	10.1, 7.8	1.98	12	0.071
Mountain Magic vs. Blank	14	1125, 559.1	67, 33	80.4, 40	31.3, 21.6	8.4, 5.8	3.33	13	0.005
Brandywine vs. Mountain Magic	14	932.1, 584	61.5, 38.5	66.6, 41.7	28, 23.6	7.5, 6.3	2.82	13	0.015
Brandywine vs. Defiant	15	925, 599	60.7, 39.3	61.7, 40	31, 26.2	8, 6.8	1.61	14	0.13
Mountain Magic vs. Defiant	14	809, 659	55.1, 44.9	57.8, 47.1	32, 31.2	8.6, 8.3	0.71	13	0.49

Table 6: First choice data for full plants of Mountain Magic, Defiant and Brandywine compared to blank air and each other.

Test	N	First choice	Chi Square	Asymptotic Significance
Full Plant				
Defiant vs. Blank	13	11, 2	6.2	0.013
Brandywine vs. Blank	13	9, 4	1.9	0.17
Mountain Magic vs. Blank	14	9, 5	1.1	0.29
Brandywine vs. Mountain Magic	14	7, 7	0	1
Brandywine vs. Defiant	15	8, 7	0.067	0.80
Mountain Magic vs. Defiant	14	6, 8	0.28	0.59

Damaged Vs. Undamaged

Damaged strains that were run against undamaged plants also yielded useful data. In the Defiant trials, bumblebees spent 47% of the 3 minute time period in the undamaged arms (p=0.68) while the bumblebees chose the undamaged arm first at a rate of 65% in 14 trials; 1.14 x^2, p=0.29 (Figure 7; Tables 7 and 8). Bumblebees did not spend more time in the arms of the Y-maze containing undamaged Brandywine VOCs (56.6% of time spent in undamaged arms) when compared with damaged BW, but the unharmed plants were chosen first significantly more often at 91.7% of 12 trials; 8.33 x^2, p=0.004 (Figure 7; Tables 7 and 8). Bumblebees spent 54% of the total time in undamaged MM

arms (p=0.57), while the first choice was split at 50% between the undamaged and damaged plants in 14 trials (Figure 7; Tables 7 and 8).

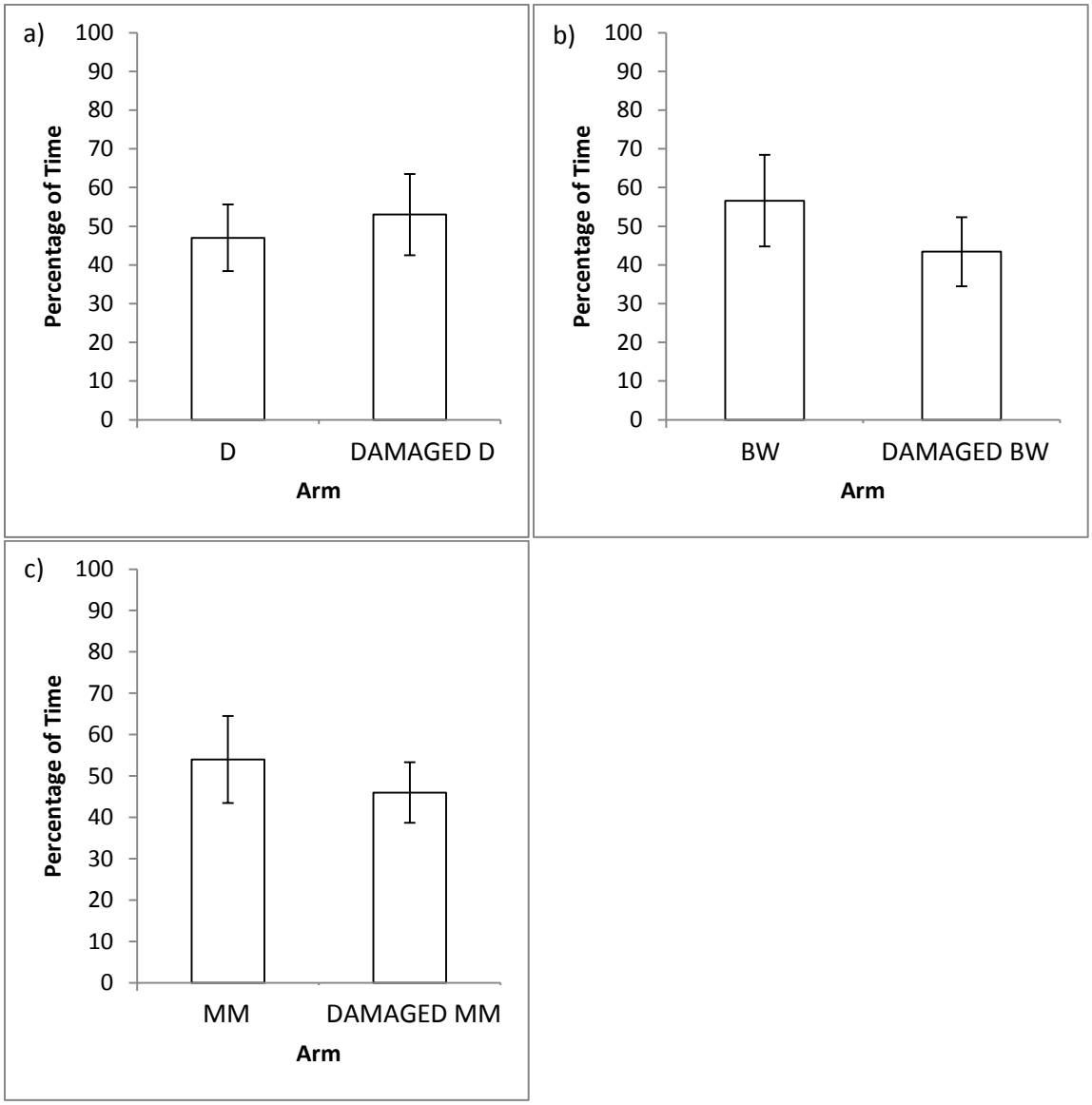

Figure 7: Damaged versus undamaged plants of the same strain (full plants): Amount of time spent in each arm of the Y-maze as a percent of total time. Part a) refers to the comparison of damaged and undamaged Defiant, b) refers to the comparison of damaged and undamaged Brandywine and c) refers to the comparison of damaged and undamaged Mountain Magic. The error bars represent ± standard error.

Table 7: Data comparison for the amount of time that bumblebees spent in the Y-maze arms containing VOCs of the damaged and undamaged plants of Mountain Magic, Defiant and Brandywine strains. DF is an abbreviation for degrees of freedom.

Test	N	Total Time in Each Arm (s)	Percentage of Time in Each Arm	Mean (s)	Std. Deviation	Std. Error Mean	T-value	DF	Significance (2 tailed)
Undamaged vs. Damaged									
Mountain Magic	14	882.3, 750.8	54, 46	63, 53.6	39.3, 27.4	10.5, 7.3	0.58	13	0.57
Defiant	14	803.5, 906.7	47, 53	57.4, 64.8	32.3, 39.2	8.6, 10.5	0.43	13	0.68
Brandywine	12	876.9, 672	56.6, 43.4	73.1, 56	40.7, 30.7	11.8, 8.9	0.93	11	0.37

Table 8: Comparison of first choice behavior in bumblebees when presented with damaged and undamaged plants of the same strain.

Test	N	First choice	Chi Square	Asymptotic Significance
Undamaged vs. Damaged				
Mountain Magic	14	7, 7	0	1
Defiant	14	9, 5	1.14	0.29
Brandywine	12	11, 1	8.33	0.004

Results of B-phellandrene Amounts

Using Newton's method, the amount of β-phellandrene in each air sample was calculated. Brandywine, the least disease resistant strain, released the most β-phellandrene (Figure 8).

Rankings	Number	Amount of B-phellandrene in Ng's
Damaged Brandywine	9	12660.05
Brandywine	7	9471.43
MM damaged	3	5899.53
Brandywine cutting	8	2034.05
Mountain Magic	1	834.53
Defiant	4	742.48
Defiant Damaged	6	463.92
Defiant cutting	5	356.04
Mountain Magic cutting	2	58.14
Control	10	29.56

Figure 8: Rankings, from largest to smallest, of β-phellandrene amounts for each cultivar, cutting, and damaged plants. Quantities were obtained using Newton's method.

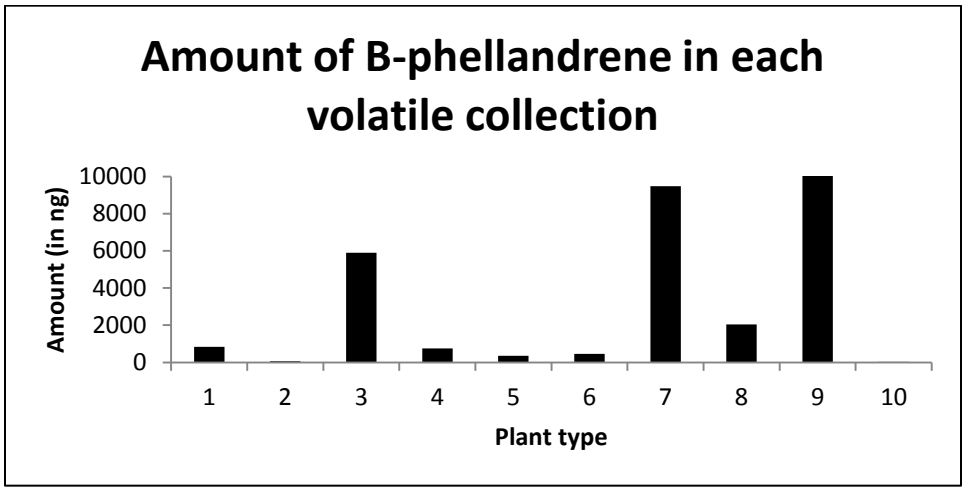

Figure 9: Graphical representation of the quantity of β-phellandrene in each volatile profile. 1-3 represents Mountain Magic (whole plant), Mountain Magic cutting and damaged Mountain Magic Plants., 4-6 represents Defiant in the same order, and 7-9 represent Brandywine. 10 is the blank control.

Jasmonic Acid

When run against blank air (with Q-tip control), bumblebees did not spend more time in the Y-maze arms containing jasmonic acid; p=0.43 (Table 9). The bees spent 55.2% of the total time in the acid containing arm and 44.8% of time in the blank arm (Figure 10, Table 9). The 20 bees chose the jasmonic acid containing arm 13 times and the blank arm 7 times for a chi square value of 1.8, p=0.18 (Table 10).

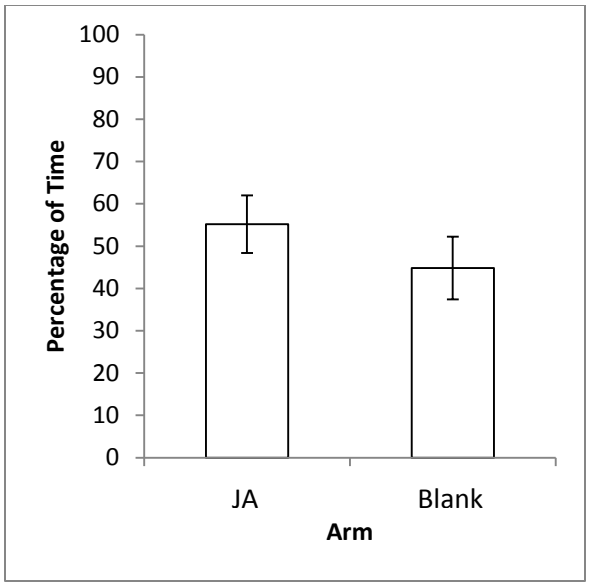

Figure 10: Amount of time (in percent) that bumblebees spent in each arm of the Y-maze during the jasmonic acid and blank treatment. The error bars represent ± standard error.

Table 9: Data comparison for the amount of time bumblebees spent in the jasmonic acid arm and blank air arm of the Y-maze.

Test	N	Total Time in Each Arm (s)	Percentage of Time in Each Arm	Mean (s)	Std. Deviation	Std. Error Mean	T-value	DF	Significance (2 tailed)
Jasmonic Acid Vs. Blank	20	895.7, 726.6	55.2, 44.8	44.8, 36.3	30.2, 33.1	6.8, 7.4	0.8	19	0.43

Table 10: Comparison of first choice behavior in bumblebees when presented with jasmonic acid and blank air.

Test	N	First choice	Chi Square	Asymptotic Significance
Jasmonic Acid Vs. Blank	20	13, 7	1.8	0.18

Discussion

Although bumblebees did not prefer the volatiles of any of the three strain cuttings when compared to blank air, the bumblebees were significantly more attracted to Brandywine, the heirloom plant, than Mountain Magic, the most disease resistant. The same result occurs in the full plant trial; the bumblebees significantly preferred Brandywine over Mountain Magic. This finding generates the hypothesis that artificial selection may inadvertently take out or reduce attractive traits in a plant; in this case, volatile emissions. Therefore, it may be possible that the more a strain undergoes artificial selection for disease resistance, the more likely that an attractive trait such as the amounts of certain effusive volatiles is decreased or lost. This point is further supported in the damaged versus undamaged plant tests. Bumblebees were not differentially attracted to the undamaged or damaged plants of any strain, however, the first choice that the bees made were different across all three cultivars. Bees showed a significant first scent attraction to undamaged Brandywine when exposed to a damaged counterpart; there must be a VOC variation. Mountain Magic, on the other hand, showed no variation in pollinator attraction, supporting the idea that some volatile trait has been lost or decreased.

The difference in β-phellandrene release across all three strains raises questions about its effect on pollinator attraction. Brandywine, the most attractive strain and least disease resistant, had the most β-phellandrene. However, Morse and others (2012) state that β-phellandrene and 2-carene may be anti-herbivory volatiles, and that plants reduce their production of these volatiles during peak pollination periods to facilitate pollination.

Therefore, with these conflicting results, it is necessary to conduct an olfactory-based behavioral test to determine β-phellandrene's effect on pollinators. It is possible that pollinator attraction is determined by a threshold that, if crossed, facilitates pollination: the high levels of attraction to Brandywine may be a result of this threshold. However, it was not possible to test β-phellandrene in my studies because of the cost ($2000/g) and the difficulty in synthesizing the chemical. A compound by compound study of pollinator attraction may help determine which volatiles are the most important to facilitating pollination, and therefore, may help agricultural companies easily and cost-effectively determine the tradeoffs between conferring disease resistant traits, and modifying volatile quantities.

Bumblebees did not spend more time in the arms containing jasmonic acid when compared to blank air in the Y-maze assay. The first choice of the bees was also not significant. However, these results support my previous findings that bees do not avoid cuttings and damaged plants based on simulated herbivory, which releases jasmonic acid. Through an olfactory based Y-maze behavioral apparatus it is possible to determine pollinator behavior, and therefore, preference, when exposed to plant tissue, whole plants, and damaged plants. The marked ability of bumblebees to choose between strains through olfaction in a laboratory setting is the groundwork for future studies. I have documented that in a Y-maze, bumblebees are more attracted to heirloom plants than highly disease-resistant plants when they choose solely based on olfaction. These findings open the door for future studies using additional strains of plants and other behavioral choice tests. It is necessary to maintain this work as agricultural companies

continue to create new strains every year. The consequences of artificially selecting for disease resistance may be detrimental to pollinator attraction in many more cultivars than those used in this study, even those widely used in agriculture. The loss of pollinator attraction through increased plant resistant traits could potentially affect crop yield, which may significantly impact how companies decide which strains to cross, and which traits to add or drop in new strains. These results underline how interconnected genetics, ecology and animal behavior are, and raise more questions surrounding the myriad ecological effects of artificial selection on agriculture.

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